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(54) Title: METHOD OF PREPARING OLIGONUCLEOTIDE PROBES OR PRIMERS, VECTOR THEREFOR AND USE THEREOF

(57) Abstract

A method of preparing a nucleic acid sequence capable of hybridizing to a target DNA sequence without requiring knowledge of this DNA sequence comprises the steps of ligating or otherwise linking a DNA fragment related to the target DNA sequence to a nucleotide sequence containing the recognition motif of an asymmetrically cleaving restriction enzyme, and subjecting the construct to the restriction enzyme to thereby cleave the construct within the target-related DNA fragment part thereof. A vector therefor comprises a site for insertion of the target-related DNA fragment, and a recognition sequence for an asymmetrically cleaving restriction enzyme or enzymes on one or both sides of said insertion site.

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METHOD OF PREPARING OLIGONUCLEOTIDE PROBES OR PRIMERS, VECTOR THEREFOR AND USE THEREOF

FIELD OF THE INVENTION

The present invention relates to the preparation of nucleic acid probes and primers as well as vectors therefor.

BACKGROUND OF THE INVENTION

The polymerase chain reaction, or PCR, provides a highly efficient method of isolating a desired gene sequence(s) from different DNA samples. One general requirement is that sequence information is available from both ends of the fragment to be amplified in order to synthesize specific amplification primers. The expense of sequencing DNA and then chemically synthesizing primers still limits the scope of many applications. In many investigations it would therefore be most helpful if the steps of DNA sequencing and oligonucleotide synthesis could be avoided entirely before genomic fragments are amplified by PCR. This is true for instance for the large programs designed to analyze the degree of polymorphism of specific DNA segments in individuals in a population, in order to identify polymorphic markers in the human or other genomes. Such genetic markers are required for mapping genetic disease and to establish or extend genetic linkage maps for a variety of organisms.

One approach which partially overcomes the above problem is the use of relatively nonspecific amplification with a limited set of primers under nonstringent conditions, so called RAPD markers. These markers serve as a means to establish relatively random polymorphic markers, analyzable by PCR without requiring DNA sequencing and chemical oligonucleotide synthesis of specific primers.

SUMMARY OF THE INVENTION

The present invention provides a different strategy to overcome the deficiences of the prior art methods and permits enzymatic synthesis of specific amplification primers, ligation probes or other probes of known structure

but partially unknown sequence without access to clone specific DNA sequence information.

In one aspect, the invention provides a vector for the preparation of a nucleic acid sequence of known structure but partially unknown sequence, capable of hybridizing to a target DNA sequence without requiring knowledge of this DNA sequence, which vector comprises a site for the insertion of a DNA fragment related to the target DNA sequence, and a recognition sequence for an asymmetrically cleaving restriction enzyme or enzymes on one or both sides of said insertion site.

In another aspect, the invention provides a method of preparing a nucleic acid sequence of known structure but partially unknown sequence, capable of hybridizing to a target DNA sequence without requiring knowledge of this DNA sequence, which method comprises the steps of ligating or otherwise linking a DNA fragment related to the target sequence to a nucleotide sequence containing the recognition motif of an asymmetrically cleaving restriction enzyme, and subjecting the construct to the restriction enzyme to cleave the construct within the DNA fragment part thereof.

In still another aspect, the invention provides a method for amplifying a DNA fragment without requiring knowledge of the DNA sequence of the fragment by using enzyme-synthesized primers prepared according the above aspect of the invention.

In yet another aspect, the invention provides a method for constructing probes for target-dependent ligation reactions.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description thereof which includes an illustrative example of the practice of the invention.

The invention will be described in more detail below with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B are a schematic representation, in the form of four parts A-D, of a strategy to derive amplification primers enzymatically. Part A shows the construction of a specialized cloning vector, part B shows the cloning of an AluI fragment into the vector, part C shows enzymatic synthesis of amplification primers, and part D shows amplification of sequences corresponding to those cloned in the vector using primers derived from the amplified insert of the clone. The recognition sequences of the restriction enzymes GsuI and AluI are highlighted in the figures.

Fig. 2 is a schematic representation of an immobilized vector containing a GsuI site, the cleavage thereof to prepare a circularizable probe, and the use of the latter for the capture and subsequent release of single-stranded circular target molecules.

Fig. 3 is a photograph of an autoradiograph of a comparison of amplification products obtained by PCR using sets of primers derived by solid-phase chemical synthesis or enzymatically. For an AluI fragment of a known DNA sequence, specific 16-mer primers that were synthesized by standard solid phase chemistry, were compared with similarly constructed 38-mer primers of the same 16-base sequence at the 3'-end, and with an enzymatically synthesized primer pair, expected to have the same 38-mer sequence as the chemically synthesized oligonucleotides of this size.

DETAILED DESCRIPTION OF THE INVENTION

A restriction endonuclease, or restriction enzyme, is an enzyme capable of recognizing a specific DNA sequence (usually four to eight nucleotides in sequence) and cleaving the DNA, thereby creating double-stranded breaks. The restriction enzymes are grouped into four classes, type I, II, III and IV, on the basis of their subunit structure, cofactor requirements, substrate specificity and several other features. Type I enzymes cleave the substrate DNA almost at random at a great distance from the recognition

site. Type II enzymes, exemplified by EcoRI, cleave within the recognition site or a few nucleotides away. Both type III and type IV enzymes cleave the target DNA sequence some considerable distance away from the recognition site and they also have in common the property of never cleaving the DNA to completion. Type IV enzymes differ from the type III enzymes in that they, similar to the type II restriction enzymes, are not dependent upon ATP. The type III and type IV restriction enzymes are commonly called asymmetrically cleaving enzymes. Exemplary of type IV restriction enzymes are GsuI (Janulaitis et al., Nucl. Acid Res. 1989; 17(14):1989), with the recognition motif 5'CTGGAG3' and cleaving 16 bases downstream of this motif; Eco57I, with the recognition sequence 5'CTGGTG3' and cleaving 16 bases downstream of this motif; MmeI (Boyd et al., Nucl. Acid Res. 1986; 17(14):5255-5274, with a 6-base recognition motif, and which cleaves the target DNA 22 bases downstream of this sequence. Restriction enzyme BpmI (available from New England Biolabs, U.S.A.) has the same recognition sequence and cleaves in the same way as GsuI above. There are also type III or IV enzymes cleaving the target DNA even farther from the recognition sequence. Examples of such enzymes include Eco15 and HinP151 cleaving 25 or 26 bases downstream of the recognition sequence.

According to the present invention, the property of type III or IV restriction enzymes to cleave asymmetrically, i.e. to cleave a DNA substrate at a distance from the recognition sequence, is utilized to prepare probes or primers of a defined size capable of hybridizing to a target DNA sequence without a requirement to know this DNA sequence.

In one embodiment of the invention, amplification primers are prepared which may then be used, optionally in combination with a standard vector sequence-derived oligonucleotide, for amplification to large copy numbers, starting from a genomic DNA sample.

Thus, defined size amplification primers may be produced enzymatically through digestion inside the cloned

fragments in amplification products by locating a recognition sequence for an asymmetrically cleaving restriction enzyme on both sides of the cloning site. The digestion is independent of the DNA sequence of the cloned fragment, and the cleavage products consisting of the original amplification primers having linked thereto a respective part of the cloned DNA fragment may be used to amplify sequences corresponding to the cloned DNA fragment from other DNA samples. Such a procedure for producing amplification primers will now be described in more detail.

With reference to Fig. 1A, part A, a plasmid vector is cleaved at a desired site, and an oligonucleotide duplex, or linker, containing the recognition site for an asymmetrically cleaving restriction enzyme, in the figure by way of example the recognition motif for GsuI, is ligated to each vector fragment end. Both linkers lack 5'-phosphate groups to prevent them from ligating to themselves. Ligation of linkers to the restriction fragment ends does not recreate the restriction site, so that over time more and more restriction ends are modified with linker dimers. The vector produced is then isolated, e.g. by gel purification, from remaining circular vector molecules and used for molecular cloning of DNA fragments. This is schematically illustrated in Fig. 1A, part B which shows the construction of a recombinant molecule by ligating a blunt end AluI-cleaved DNA-fragment into the vector.

In order to produce amplification primers, individual clones are amplified using a single primer corresponding to one of the linker oligonucleotides and with the 5'-end protected against λ exonuclease digestion, e.g. by a biotin residue at the 5' end. The resulting amplification products consist in the cloned AluI-fragment surrounded by vector-derived sequences representing the oligonucleotide dimer ligated to the vector as described above. With reference to part C of Fig. 1A, the 6-base motif, 5'CTGGAG3', immediately flanking the insert cloned into the vector, represents the recognition sequence for the restriction

enzyme GsuI. As mentioned above, this enzyme cleaves the DNA molecule 16 bases downstream of this motif, inside the fragment cloned into the vector. After digesting the amplification products with this enzyme, the fragments obtained are separated by gel electrophoresis. Thereby the fragments derived from both ends of the amplification product may be quantitated and isolated from remaining undigested or partially digested molecules. Finally, any DNA strands that have 5' phosphate groups are removed by digestion with the enzyme λ exonuclease, whereas the extension products containing 5' biotin are insensitive to the exonuclease over a wide range of nuclease concentrations. In this manner, pairs of single-stranded amplification primers are obtained, which consist of a 5' end part, derived from the linker sequence that was added to the cloning vector by linker ligation, and a 3' end part, derived from ends of the inserts cloned into the vector. In the illustrated case (Fig. 1A, part C) there is located immediately upstream of these 16 bases in the primers a dinucleotide, AG, representing the last two positions in the recognition sequence for the enzyme GsuI. These positions are also known to be present in the genomic DNA from which the cloned fragment was derived since these fragments were derived by digestion with the blunt end generating restriction enzyme AluI (Fig. 1A, part C). Each one of the enzyme-synthesized primers therefore has a number (here 18) of bases (the 3' end bases plus two of the 5' end bases) that are complementary to the genomic sequence at each side of the cloned fragment, and a number of bases derived from the vector.

The above enzymatically synthesized primers may be used for PCR amplification of a fragment obtained by AluI digestion of chromosomal DNA as schematically illustrated in Fig. 1B, part D. First, the primers are used in a number of, say 5, amplification cycles, when necessary using a long annealing time to compensate for relatively low concentration of enzyme-synthesized primers. In these first cycles, the desired DNA fragments are obtained, flanked at

either end by the vector sequence. It is then no longer necessary to use the enzymatically synthesized primers, but the further amplification of the fragments to large numbers may conveniently be performed with the "standard" vector-derived primer, i.e one of the linker primers or a variant thereof.

In a variant of the above procedure, a cloning vector is prepared which contains the two recognition sites for the asymmetrically cleaving enzyme immediately adjacent each other but in opposite orientations and together containing the recognition site for a bluntly cleaving restriction enzyme, such that the enzyme may cleave right between the two sites. This permits the cloning vector containing the two recognition sites for the asymmetrically cleaving enzyme to be amplified prior to blunt cleavage between the sites, phosphatase treatment to prevent self-ligation of the free vector ends, and insertion and ligation of the target DNA sequence to be subsequently cleaved by the asymmetrically cleaving enzyme. As an example may be mentioned two juxtaposed but opposed GsuI (= BpmI) sites which can be bluntly cleaved between them by the restriction enzyme Ecl136II (New England Biolabs, U.S.A.)

As appears from the above, the steps of the present procedure are simple and may easily be performed for a large number of fragments in parallel, permitting the development of large sets of genetic markers. If desired, successful markers may be sequenced and converted to regular chemically synthesized PCR-based primers, but it is also possible to continue using enzyme-synthesized primers.

Enzymatically synthesized amplification primers, constructed as described above, can also provide highly specific and sensitive detection reagents. Thus, in another embodiment of the invention, amplification primers suitable to detect individual organisms in complex mixtures by PCR may be obtained by restriction digesting DNA preparations, cloning the fragments into the specialized vector described above, and isolating individual clones in order to generate

a desired number of pairs of amplification primers. These amplification primers may thus be developed and tested without any requirement for access to DNA samples from isolated organisms or for DNA sequence information from these.

In order to avoid the risk of contamination by another fragment, amplifiable with the same standard primer, precautions such as the inclusion of a ribose in the 3' position of the standard primer, rendering amplification products cleavable by alkali, may be contemplated.

It is readily seen that the same DNA fragment may be cloned into two different vectors with different linkers, or "standard primers", permitting so-called nested PCR to be performed. This is possible by using different flanking sites for enzymes that cleave at variable distances internal to the cloned fragment. In nested PCR, a number of cycles are conducted with a first set of primers, whereupon a number of cycles are run with a second set of primers hybridizing internally of the first primer set. This increases the efficiency and selectivity of the PCR process.

As is readily realized, it is essential in the preparation of the synthesized primers to isolate the desired end fragments obtained after cleavage with the asymmetrically cleaving enzyme from any remaining partially digested or undigested amplification products as incomplete digestion products may give rise to false positives in the subsequent amplification, when using the enzymatically derived primers. In the preparation procedure described above, gel electrophoresis is used to isolate the desired fragments. However, preparative gel electrophoresis is relatively cumbersome and may also fail to adequately resolve end fragments and incomplete digestion products. An alternative means of removing middle piece-containing fragments away from the desired end fragments is described below.

Despite the fact that the middle segments are of unknown sequence composition, end fragments may be

efficiently isolated by amplifying the cloned fragments in two separate reactions as follows:

In each of the two amplifications of the cloned inserts, one or the other amplification primer is biotinylated, permitting the amplified molecules to be isolated on a solid support, such as streptavidin-coated combs, e.g. of the type described in WO 94/11529, or paramagnetic particles. Other means of immobilization including DNA ligation may also be used. After adequate washes, one or the other of the end fragments are released from the rest of the amplified molecules by selectively digesting the asymmetric restriction enzyme site located at the end remote from the support. This selective digestion may be achieved by using vectors with recognition sequences for two different asymmetric restriction enzyme sites, e.g. type IV enzymes, on each side of the cloned inserts and cleaving the immobilized, amplified molecules in two separate reactions. Examples of such pairs of type IV restriction enzymes are GsuI and Eco57I.

Alternatively, vectors with a single restriction site on both sides of the insert may be used if the biotinylated amplification primers are designed such that they are mismatched to the vector in e.g. positions 5 and 6 in from the 3' end of the primer, corresponding to the first 2 bases of the 6 base recognition sequence of a type IV restriction enzyme. Other sequence alterations are, of course, also possible, preserving the ability of the primers to amplify the cloned fragment but destroying the recognition sequence for one or the other of the sites. In this strategy, the two amplification products may be pooled before immobilization on the supports and restriction cleavage. After cleavage, released fragments are treated with λ-exonuclease to obtain single stranded probes, and may then be directly used as enzymatic amplification primers.

The enzymatic primer synthesis approach of the invention may also be used for producing circularizable probes. The preparation and use of circularizable probes

(so-called "padlock" probes) have been described by Landegren et al., Science 265, 2085-2088 (1994). Such probes consist of two target-complementary segments, connected by a linker sequence. Upon recognition of a specific nucleic acid molecule, the ends of the probe are joined through the action of a ligase, creating circular DNA molecules catenated to the target sequence. These probes provide highly specific detection with minimal background.

In accordance with another embodiment of the invention, such a circularizable probe may be prepared by cloning a DNA sequence into a specially designed vector, and the vector may then be used as a circularizable probe without the use of DNA sequence information from the cloned fragment, or the use of specific oligonucleotides. A large set of such clones may be processed in parallel for e.g. subtractive comparisons between cDNA populations. For example, library to library comparisons may be made between for instance a library of all genes expressed in a given tissue and on the other hand all sequences located in a particular region of the genome, from yeast artificial chromosomes, subcloned in a circular single stranded plasmid library. This is, for example, useful when a disease gene has been located to a region by linkage analysis and it is known to be expressed in a particular tissue.

The necessary vector may be prepared by ligating oligonucleotides to a restriction digested plasmid to generate, on one hand, a recognition motif for an asymmetrically cleaving enzyme, such as GsuI, and, on the other hand, a nearby situated rare restriction site, such as NotI. One way of constructing the vector is by inserting two oligonucleotide dimers to two different ends, generated by digestion with two restriction enzymes.

Recombinant molecules are then produced by ligating blunt end DNA fragments, e.g. generated by AluI digestion, to the vector. The constructs obtained are transformed into

bacteria and single-stranded recombinant molecules are generated.

These circular single-stranded molecules are then immobilized in patches on a solid phase, e.g. a membrane, as schematically illustrated in Fig. 2. A primer is hybridized to the vector molecule and extended using a DNA polymerase, rendering the recognition sequence for the asymmetrically cleaving enzyme double stranded and permitting cleavage of the insert. After digestion of the molecules with the enzyme, the fragments of extended molecules are removed by denaturing washes.

The immobilized circularizable probes obtained may be used for trapping target molecules, as is also illustrated in Fig. 2. To this end, a population of recombinant clones prepared as above is turned into single-stranded circular molecules. For comparisons between directionally cloned cDNA populations, this library, should, of course, be of the opposite polarity to the above construct. Further, the vector must not have the same rare-cutter enzyme site or at least not the same polarity of the sequence surrounding the recognition sequence. These molecules are now exposed to the immobilized probes and trapped by ligation, followed by stringent washes.

An oligonucleotide is then annealed to the region of the rare-cutter side on the capture vectors, and these are linearized by digestion with the rare cutter enzyme. Trapped clones are released by denaturing washes, repaired, and transformed into bacteria for molecular cloning.

Since the above described approach is based on an intramolecular reaction (ligation), large numbers of clones may be treated and then applied as capture probes in the same reaction. In contrast, e.g. PCR will only permit a handful of reactions to be performed in the same reaction due to the problem of nonspecific crosstalk between primers.

In the following, the invention will be illustrated by a non-limiting example.

EXAMPLE

Chemical oligonucleotide synthesis

The following oligodeoxynucleotides were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer (Applied Biosystems Inc., USA):

- Omni1: 5' AATTG ACCGT TAGCA ACTGG AG 3'
- Omni2: 5' CTCCA GTTGC TAACG GTC 3'
- BOmni1: Omni1 above syntesized with the addition of a 5' biotin residue.

(In the sequences for Omni1 and Omni2 the recognition sequence for the restriction enzyme GsuI has been underlined.)

- Control primer A1: 5' AATTG ACCGT TAGCA ACTGG AGCTC AGCAG CCCGT GAT 3'
- Control primer AM1: 5' CTC AGCAG CCCGT GAT 3'
- Control primer A2: 5' AATTG ACCGT TAGCA ACTGG AGCTT CCAGA GTCAG ATC 3'
- Control primer AM2: 5' CTTCC AGAGT CAGAT C 3'.

Construction of the cloning vector pRIMER

Plasmid pBLUESCRIPT M13 was digested with EcoRI and, in the same reaction, the oligonucleotide duplex Omni1 and Omni2 were ligated to the restriction fragment ends (Fig. 1A, part A). 20 U of EcoRI were added to 5 µg of the plasmid in a final volume of 10 µl containing 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM potassium acetate, and 5 mM ATP. After 3 hours at 37°C, 2.5 U of T4 DNA ligase were added with oligonucleotides Omni1 and Omni2 at 25 µM each. The reaction was left at 14°C over-night. Linearized vector molecules with added linkers were gel purified in a 1.5% NuSieve GTG agarose gel (Nalgene), and used as a cloning vector.

Cloning of Alu-digested fragments and screening for positive clones

AluI-digested fragments from the human ameliogenin gene located on the X chromosome were ligated into the above vector and introduced in strain DH5 of E. coli (Fig. 1A, part B). Individual bacterial clones were transferred with a toothpick from the agar plate to 100 µl of water in

an Eppendorf tube, the samples were boiled for 5 minutes, and then chilled on ice. Cell debris was pelleted in a short centrifugation and one µl of the supernatant was used as a source of template in a 30 µl PCR with the single primer BOmni1 at 1 µM. 10 µl aliquots of the PCR samples were separated in a 1.5% agarose gel to quantitate and determine the size of the amplification products before the inserts were used for synthesis of amplification primers.

Enzymatic synthesis of specific amplification primers

In order to prepare amplification primers (Fig. 1A, part C), 2 µg of inserts that had been amplified with BOmni1 were gel purified in 2% NuSieve GTG agarose (Nalgen) and digested with 8 units of GsuI (Fermentas, Vilnius, Lithuania) in 15 µl of 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol, and BSA at 0.1 µg/µl for 3 hours at 37°C.

The digestion products were separated by electrophoresis in a 2% agarose gel. End fragments released from the amplification products by enzymatic digestion were isolated from the gel, taking care not to contaminate the material with undigested material.

Isolated fragments were treated with λ exonuclease (Life Technologies) destroying DNA strands with 5'-phosphate groups but sparing strands with a biotin residue at the 5'-position. The reactions were performed in a final volume of 10 µl, containing approximately 200 ng of end fragments, 50 ng/µl BSA, 2 units of λ exonuclease, 67 mM glycine-KOH, pH 9.4, 2.5 mM $MgCl_2$ for 1 hour at 37°C. The DNA was preheated for 5 minutes at 94°C before the reaction. Remaining 5'-biotinylated DNA strands were used as amplification primers.

Amplification using enzyme-synthesized primers

Enzymatically synthesized primers were used at a concentration of 0.05 µM, together with oligonucleotide Omni1 at 1 µM, for amplification of human genomic DNA samples (Fig. 1B, part D). During the first 5 amplification cycles, annealing times were set at 10 minutes followed by a regular amplification protocol. Since the melting

temperatures are not known for the template-specific 3'-ends of the amplification primers, two different different amplification programs were tried for each primer pair. The complete amplification program was as follows: 5 cycles of 94°C for 1 minute, annealing at 40°C or 55°C for 5-15 minutes, and 72°C for 1 minute, followed by 30 cycles of 94 °C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute.

The autoradiograph in Fig. 3 illustrates agarose gel electrophoresis of the results of amplification reactions using either chemically synthesized, 16 bases long primers (Am1 and Am2), lane 2 and lane 3 (without template); the same 16 bases with additionally 22 bases at the 5' ends identical to Omni1 (A1 and A2), lane 4 and lane 5 (without template); amplification with BOmni1 from the plasmid clone used to generate the primers, lane 6 and lane 7 (without template); or enzyme-synthesized primers for a 200 bases long AluI fragment of known DNA sequence, assumed to be identical in sequence to oligonucleotides A1 and A2, lane 8 and lane 9 (without template). Lanes 1 and 10 are 100 bp ladders. As appears from the autoradiograph, all three pairs of oligonucleotides, when applied to human genomic DNA samples, gave rise to amplification products of approximately the same size. This is the size expected for these primers. In the absence of added template DNA, no amplification products were observed, affirming that the amplification products did not represent copies of contaminating undigested amplified clone inserts.

Identification of polymorphic sequences

The amplification primer Omni1 was kinased with ^{32}P in a 30-minute reaction at 37°C in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, and 0.5 ICI $\gamma^{32}P$ ATP. The primer was used for amplification of genomic DNA samples, followed by separation of the products by 6% polyacrylamide denaturing gel electrophoresis. Size variation among amplification products was revealed by autoradiography.

CLAIMS

1. A method of preparing a nucleic acid sequence capable of hybridizing to a target DNA sequence without requiring knowledge of this DNA sequence, **characterized** in that the method comprises the steps of ligating or otherwise linking a DNA fragment related to the target DNA sequence to a nucleotide sequence containing the recognition motif of an asymmetrically cleaving restriction enzyme, and subjecting the construct to the restriction enzyme to thereby cleave the construct within the target-related DNA fragment part thereof.

2. The method according to claim 1, **characterized** in that the method comprises linking said nucleotide sequence to each end of a linearized circular vector, inserting and ligating said target-related DNA fragment into the vector, optionally multiplying said vector, and cleaving the assembly of said two nucleotide sequences and said DNA fragment from both ends within the DNA fragment by said asymmetrically cleaving restriction enzyme.

3. The method according to claim 2, **characterized** in that the method comprises the step of amplifying the assembly of said two nucleotide sequences and said DNA fragment prior to the cleavage step.

4. The method according to claim 3, **characterized** in that said-amplification is performed by enzymatic amplification using a primer hybridizing to said nucleotide sequence.

5. The method according to claim 4, **characterized** in that said primer at the 5' end thereof is protected against λ exonuclease digestion, for example by biotinylation, and that the unprotected strands obtained in the amplification process are subjected to λ exonuclease digestion.

6. The method according to claim 1, **charact riz d** in that the method comprises linking said nucleotide sequence containing the recognition motif of an asymmetrically cleaving restriction enzyme to one end of a linearized nucleic acid plasmid vector, inserting and ligating said target-related DNA fragment into the vector, and cleaving the vector within said DNA fragment by said restriction enzyme to thereby form a circularizable probe.

7. The method according to claim 6, **characterized** in that the method further comprises providing a rare restriction site near said restriction enzyme recognition motif.

8. The method according to any one of claims 1 to 7, **characterized** in that after said cleavage, the desired hybridizing nucleotide sequence or sequences are isolated by gel purification, preferably gel electrophoresis.

9. The method according to any one of claims 3 to 8, **characterized** by immobilizing the amplified fragments to a solid support prior to performing the cleavage step.

10. The method according to claim 9, **characterized** by providing a recognition sequence for an asymmetrically cleaving restriction enzyme on each side of the target-related DNA fragment, performing two amplification reactions to obtain two amplified fragments that bind to the solid support, and selectively cleaving each amplified fragment at the asymmetric enzyme restriction site located at the end remote from the support.

11. The method according to claim 10, **characterized** by providing different asymmetrically cleaving restriction enzyme sites on each side of the target-related DNA fragment, and cleaving the immobilized amplified fragments in two separate reactions.

12. A vector for the preparation of a nucleic acid sequence of known structure but partially unknown sequence capable of hybridizing to a target DNA sequence without requiring knowledge of this DNA sequence, **characterized** in that the vector comprises a site for the insertion of a DNA fragment related to the target DNA sequence, and a recognition sequence for an asymmetrically cleaving restriction enzyme or enzymes on one or both sides of said insertion site.

13. The vector according to claim 12, **characterized** in that, after insertion of said DNA fragment, the vector may be rendered single-stranded and is capable of forming a circularizable probe after cleavage by said asymmetrically cleaving restriction enzyme.

14. The vector according to claim 13, **characterized** in that it further comprises a rare restriction site near said recognition sequence.

15. The vector according to claim 13 or 14, **characterized** in that it has said DNA fragment inserted into said site and optionally is immobilized to a solid support, such as a membrane.

16. A method for amplifying a DNA fragment without requiring knowledge of the DNA sequence of the fragment, **characterized** by using as extension primers enzyme-synthesized primers prepared according to any one of claims 1 to 5.

17. The method according to claim 16, **characterized** by performing a first number of amplification cycles by said enzyme-synthesized primers, and then performing a second number of amplification cycles by chemically synthesized primers capable of hybridizing to said nucleotide sequence linked to the linearized vector ends.

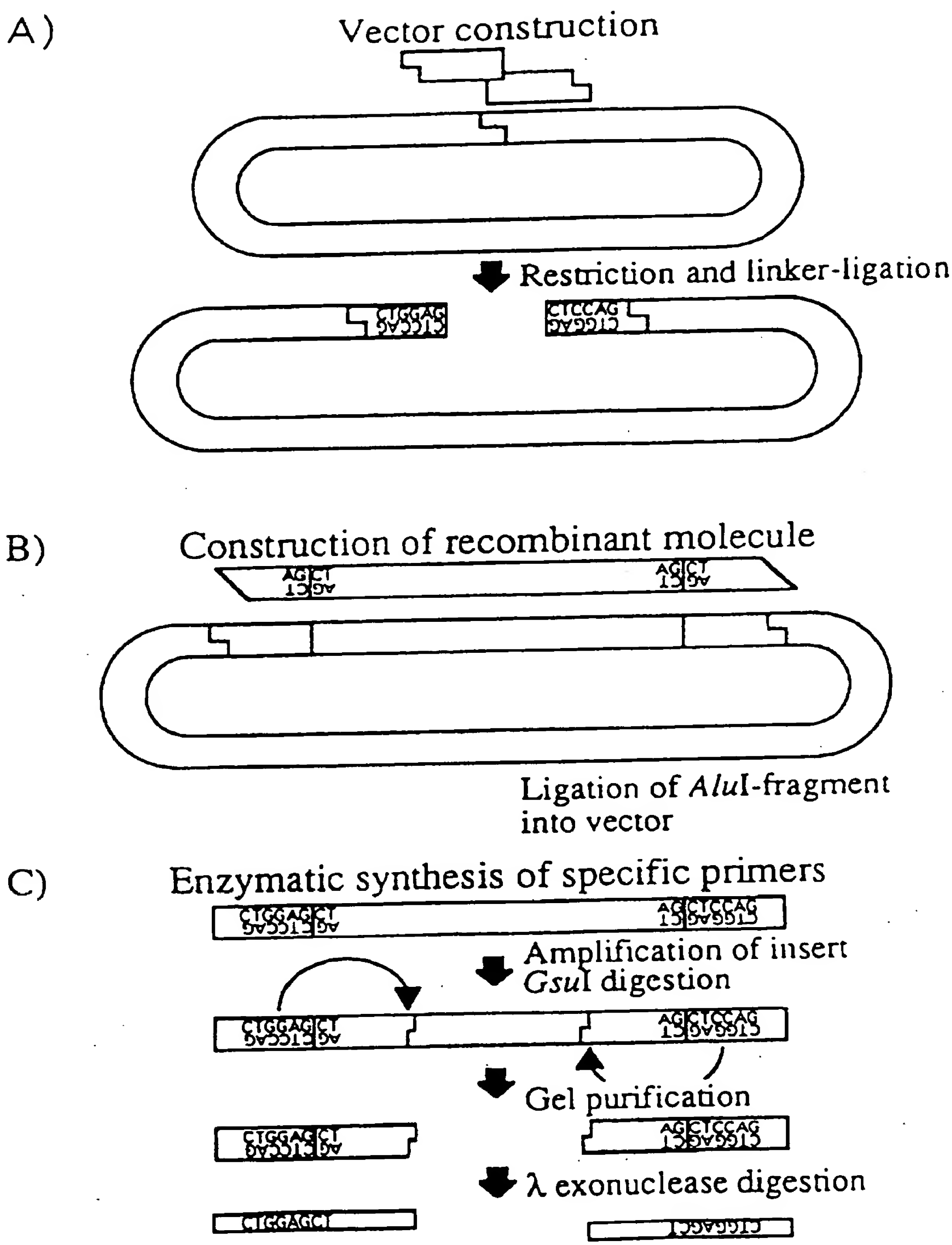
A)
Vector construction
Restriction and linker-ligation
CTGGAG
CTCCAG
B)
Construction of recombinant molecule
AG CT
Ligation of *Alu*I-fragment into vector
C)
Enzymatic synthesis of specific primers
CTGGAG CT
AG CTCCAG
Amplification of insert
*Gsu*I digestion
Gel purification
λ exonuclease digestion
CTGGAGCT

FIG. 1A

D) Amplification using enzyme-synthesized primers

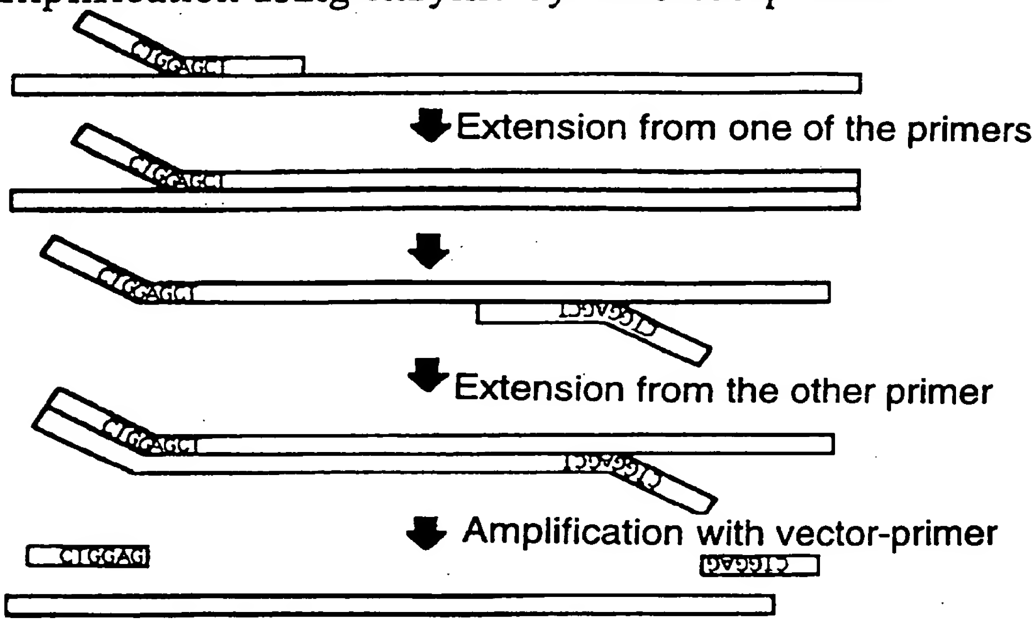

FIG. 1B

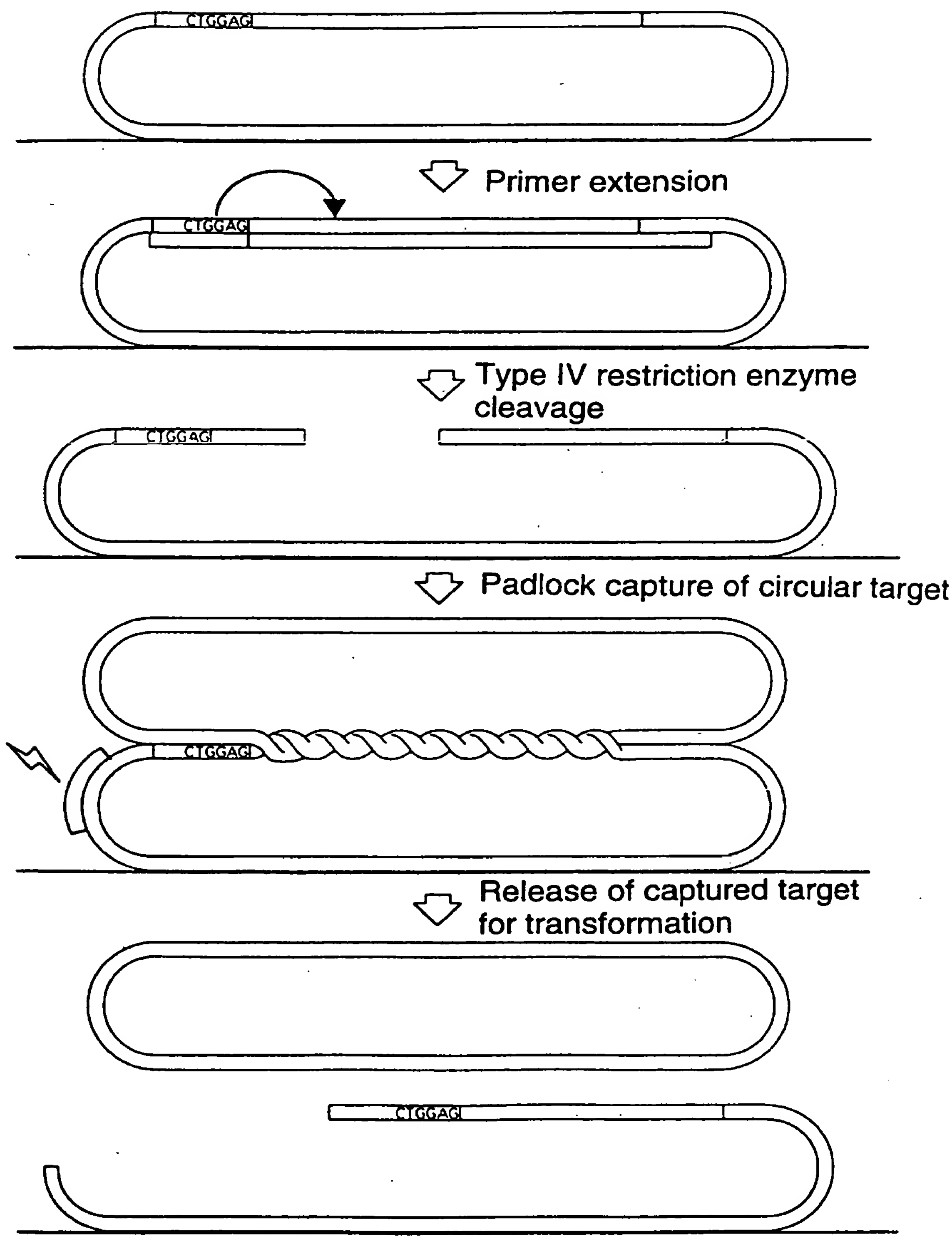
CTGGAG
Primer extension
CTGGAG
Type IV restriction enzyme cleavage
CTGGAG
Padlock capture of circular target
CTGGAG
Release of captured target for transformation
CTGGAG

FIG. 2

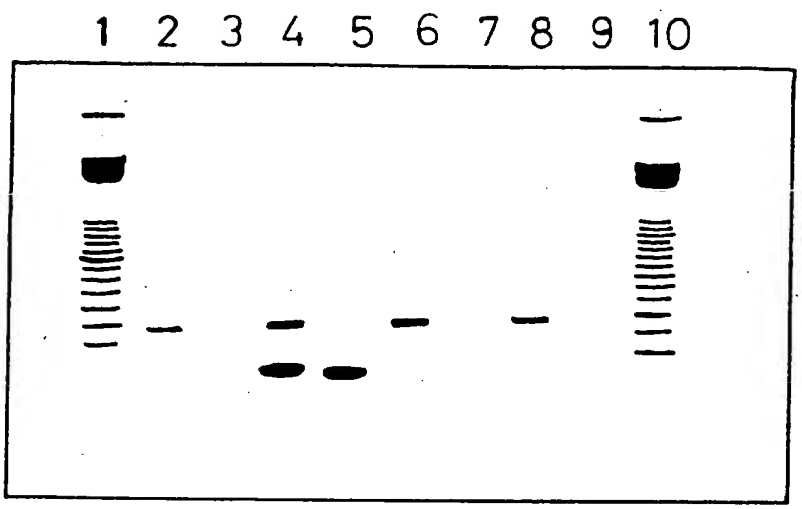

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/01319

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/10, C12N 15/66 // C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum docum ution searched (classification system followe iassification symbols)

IPC6: C12N, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EDOC, BIOSIS, MEDLINE, DBA, SCISEARCH, PATENT CITATION INDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GENE Volume 54, 1987, R.B. GAYLE III et al, ormation of MboII vectors and cassettes using asymmetric MboII linkers" page 221 - page 228 --	1-17
A	WO 9001548 A1 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED), 22 February 1990 (22.02.90) -- --------	1-17

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" erlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than t. priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 March 1996	14.03.96

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT
Information on patent family members

05/02/96

International application No.
PCT/SE 95/01319

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9001548	22/02/90	NONE	